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PRODUCTION OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS IN CELLS GRO--ETC(U)
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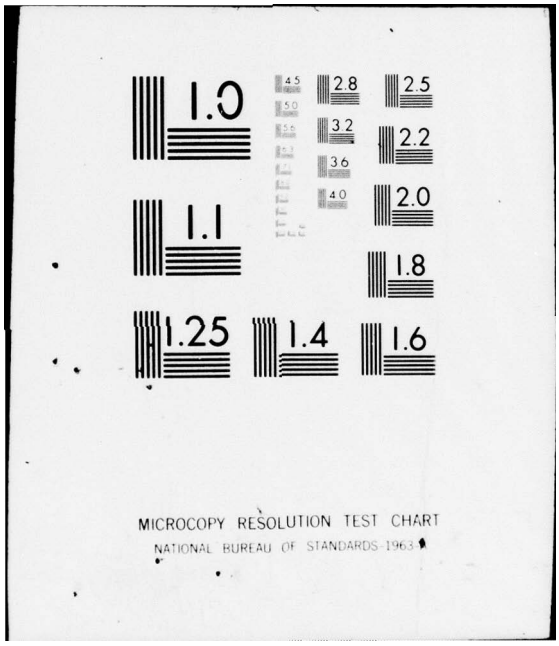
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Running Head: VEE VIRUS GROWN USING ARTIFICIAL CAPILLARIES

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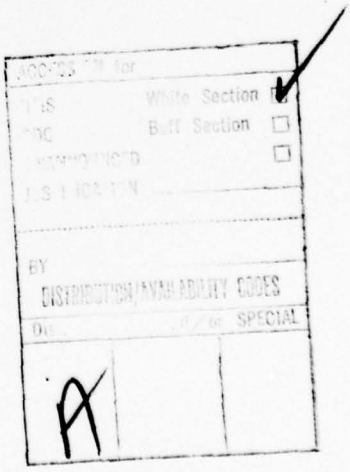

ABSTRACT

Primary cell cultures, a continuous cell line, and a diploid cell line were grown on an artificial capillary system. The cells were subsequently infected with Venezuelan equine encephalitis virus (VEE) and viral replication was studied. Extracellular fluids harvested from this system contained high titers of virus and were relatively free of cell debris.

Artificial capillary systems have been used in a limited number of experiments to study the effects of continuous perfusion of fresh medium through the cells (1, 3, 6). To a certain degree, these systems have eliminated contact inhibition and the cells in culture have attained a three-dimensional organ-like structure. Knazek et al. (3), who first reported the cultivation of choriocarcinoma cells in such a system, measured the amount of human chorionic gonadotropin (hCG) produced in an artificial capillary system and found that cells in this system produced 11 times more hCG than cells in a monolayer. Wolf and Munkelt (6) cultured rat hepatoma cells on an artificial capillary system and found that these cells were able to take up, conjugate, and excrete bilirubin. Chick et al. (1) studied beta cells from neonatal rats, attempting to establish an artificial pancreas. Their data indicated that cells cultured in this manner maintained their ability to synthesize and release insulin.

Our experiments were designed to study the feasibility of artificial capillary systems to grow cells for production of viral vaccines. Since virus yield is often directly proportional to the number of cells, an increase in total cells within a given surface area might markedly increase virus yield. The extensive surface area provided by the fiber bundles of the artificial capillary system offered promise of fulfilling this requirement.

MATERIALS AND METHODS

Artificial capillary system. The Vitafiber unit, type 300 S, manufactured by Amicon (Lexington, Mass.) was used. The capillaries are made of polysulfone, have an internal diameter of 200 μm, a

molecular weight cutoff of 100,000, and are encased in bundles of 150 fibers per unit. The fiber length is 5.7 cm, and the total area is approximately 60 cm^2. The extracapillary volume of the Vitafiber unit is 2.6 ml. Each Vitafiber unit has two access ports located on top of the unit. By positioning syringes on each of these ports, cells can be seeded onto the outside of the fiber bundle. In addition, by using syringes filled with fresh medium, samples of supernatant can be removed for examination.

As a means of providing for gas exchange, 1 m of silicone tubing was used for each Vitafiber unit. All units were housed in a 37 C incubator in an atmosphere of 90% air and 10% CO_2. An Erlenmeyer flask containing fresh medium served as a reservoir for the system. Medium was pumped through the fibers at a flow rate of 5 ml/min.

Primary chick cell culture. Embryonated chick eggs were harvested at 10 days, essentially as described by Cole et al. (2). The embryos were decapitated, then finely minced with scissors, and dispersed in trypsin. The resulting cell slurry was predominantly fibroblasts, and was used without further cell differentiation. Medium E199 (Grand Island Biologics, Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS) was used for the primary chick cell cultures. Antibiotics were added to give a final concentration of 100 units of penicillin and 100 µg of streptomycin per ml (PS).

Diploid cell line. Fetal rhesus lung cells (5) were obtained from American Type Culture Collection (Rockville, Md.), and were plated according to their directions. Cells were harvested from T-150 monolayers for use in the Vitafiber system. Eagle's minimal essential medium (Grand Island Biologics), supplemented with 10%

FCS, was used to culture the fetal rhesus lung cells.

Continuous cell line. BHK-21, a line of Syrian baby hamster kidney cells were obtained from American Type Culture Collection and were propagated in BHK-21 medium (Grand Island Biologics) with 10% FCS and PS.

Virus. Venezuelan equine encephalitis (VEE) virus was used as the attenuated vaccine strain, TC-83, which has been previously described (2).

Virus titrations. Virus suspensions were diluted in cold Hank's balanced salt solution (HBSS) containing 2% heat inactivated FCS, PS, and 10 mM HEPES buffer, pH 7.3 (standard diluent). Chick embryo cell (CEC) culture monolayers were infected with 0.2 ml of a test dilution. The adsorption period was 1 h, and the cell sheets were tilted at 15 min intervals to distribute the inoculum. The inoculated CEC culture monolayers were overlaid with Eagle's Basal Medium with Earle's salts (EBME) containing 4% heat inactivated FCS, 1% agarose, and PS. After 18 h incubation at 36 C in a humidified atmosphere of 5% CO_2 in air, the monolayers were stained with a 1:3,150 dilution of neutral red incorporated in a second overlay to increase the visibility of the plaques. The cultures were returned to the incubator and plaques were counted the following day. Virus titers are expressed as $\log_{10}$ plaque forming units (PFU)/ml.

Physical quantitation of virus. Virus particle quantitation was performed by the agar sedimentation technique of Sharp (4).

RESULTS

In preliminary experiments, several different densities of CEC were seeded onto the fiber surface of the artificial capillary system. The cells were allowed to attach for 1-2 h before medium perfusion was initiated. The cells were allowed to grow in the artificial capillary system for 3-7 days before they were infected. Cell viability was evidenced by a change in the pH of the medium; the medium in the reservoir was changed every 3 days. In the first series of experiments, the same amount of virus input was used to infect varying numbers of cells. Samples were removed from the extracapillary spaces by using 1 ml syringes filled with fresh medium. Since the total fluid space of the system is 2.6 ml, approximately 40% of the supernatant was removed at each sampling time. Samples were removed at several time intervals to determine the replication rate of the virus in the CEC growing in this system. Results from these experiments are shown in Fig. 1. Increasing the number of cells originally seeded onto the fibers resulted in an increase in total virus yield.

When the 24-h samples were examined by electron microscopy (Fig. 2), they were remarkably free of cellular debris, whereas conventional monolayers of CEC cultures at 24 h postinfection were heavily laden with large clumps of debris from viral cytopathic effect. This observation suggested that the cells might still be viable after VEE virus infection. A series of experiments was conducted to determine the level of continuous virus production. Samples were harvested daily from 1-6 days postinfection. The results of these experiments are shown in Fig. 3. By 6 days

postinfection, the virus titer was still in excess of 10^7 PFU/ml. A comparison of plaquing efficiency was performed with various samples by correlating the PFU titer with particle counts (Fig. 3). At 24 h postinfection, the sample contained 3.2×10^9 PFU/ml, and the particle count was 2.5×10^9; the plaquing efficiency (particles/PFU) at this time was 1. At 72 h postinfection, the sample contained 1.6×10^9 PFU/ml, but the particle count has risen to 5.0×10^9, increasing the particle/PFU ratio to 3. At 98 h postinfection the sample contained 8.0×10^7 PFU/ml and the particle count was 1.5×10^9; the particle/PFU ratio has risen to 20.

A heat-inactivation control was established with virus in the absence of cells in the artificial capillary system. Titers of daily samples indicated a predictable fall in viable virus particles. When compared to CEC, it appeared that the slopes of the infected culture and the heat-inactivated virus were parallel after 48 h, indicating that virus harvested after this time had been released from the cells into the surrounding supernatant, and was not newly produced.

In another set of experiments, the number of CEC seeded onto the fibers was kept constant, while the input multiplicity of virus was changed. The results (Table 1) show that an increased multiplicity of inoculum resulted in a higher yield of virus.

Several other types of cells have been used in the artificial capillary system to support virus reproduction. Primary duck embryo cells were prepared in the same manner as the chick cells, and were used successfully to grow the virus; however, there was no advantage over the CEC. A continuous cell line, baby hamster kidney (BHK-21),

and a diploid line, fetal rhesus lung, also could be used to produce virus. These results are also summarized in Table I.

DISCUSSION

Artificial capillary units have been used as substrate for culturing several types of cells. These cells have then been infected with virus and yielded relatively cell-free material with high virus titers. The small volume of the extracapillary space in the Vitafiber system, by design, produces a concentrated virus-containing supernatant. This material could be used for vaccine production, or alternatively might provide starting material for virus purification. The absence of gross cellular debris, even at several days postinfection may be suggestive of extended cell viability in this system. Conventional monolayer cell cultures, when infected with VEE in a similar fashion, are typified by extensive cytopathic effect of the virus; supernatant material is laden with large particles of cellular debris, making purification more difficult. The cleaner material obtained from the Vitafiber cultures could facilitate purification procedures.

One other potential application of this system lies in the use of the reservoir material to harvest extracellular products or soluble viral proteins. Since the molecular weight cutoff of the fibers is 100,000, many molecules could pass from the cells through the fibers to a collection point (which could be substituted for the recycled reservoir system). One obvious example of a cell-associated product which could be produced in the artificial capillary system is interferon. In addition, the continuous removal of interferon from the cell cultures might allow extended production of virus particles.

Viral subunit antigens, such as hemagglutinin or complement-

fixing antigen, could also be harvested through the fibers of this system. These soluble antigens could then be used for diagnostic purposes in serological assays.

The artificial capillary system offers several distinct advantages over conventional monolayer cultures for virus production. Each unit is compact, uses less medium than a roller bottle, and yet still provides adequate conditions for growth of cells. Supernatant fluids from VEE virus-infected cells are concentrated, and relatively free of gross cellular debris. The Vitafiber units can be reused, and during operation, the system requires little maintenance.

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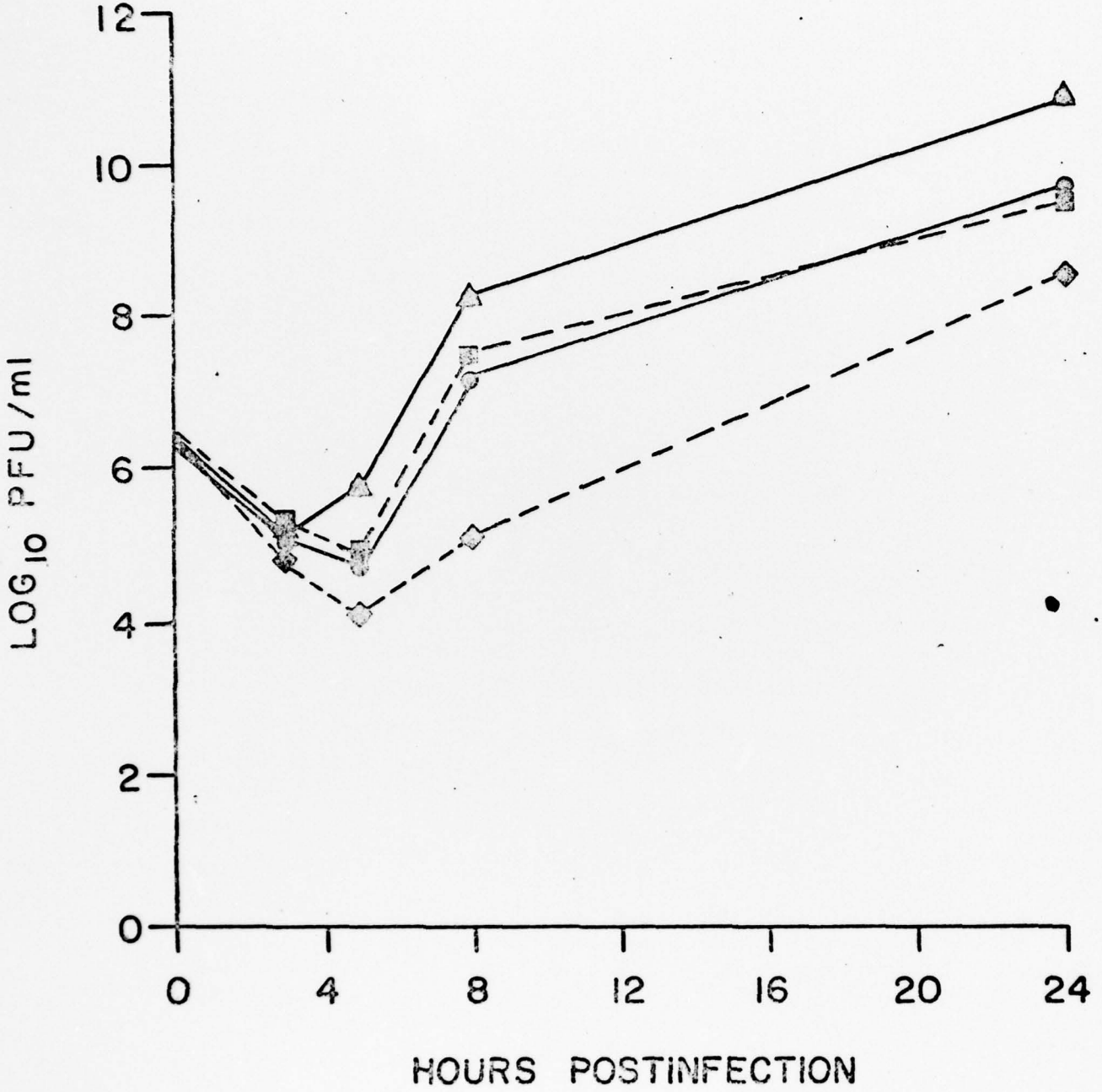
Table 1. VEE virus growth in Vitafiber unit at 24 h

Cell line	Multiplicity of inoculum	Log_{10} PFU/ml (± SE)
Primary chick	0.1	8.1 ± 0.4
	1	9.8 ± 0.4
	10	10.7 ± 0.3
Primary duck	10	10.0 ± 0.1
Bhk-21	1	9.4 ± 0.1
Fetal rhesus lung	10	10.3 ± 0.3

Figure 1. Growth of VEE virus in chick fibroblasts. Input of virus was 5×10^6 PFU for all experiments. Samples were removed periodically for virus titrations. Symbols: (◆) 3.7×10^6 chick fibroblasts; (●) 1.7×10^7 chick fibroblasts; (■) 3.7×10^7 chick fibroblasts; (▲) 1.7×10^8 chick fibroblasts.

Figure 2. Electron micrograph of supernatant from Vitafiber unit containing chick fibroblasts infected with VEE virus. Bar: 1μ.

Figure 3. Extended harvest of virus from chick fibroblasts. Samples were removed daily for virus titration and physical particle quantitation. Heat-inactivated control contained virus in complete medium in a Vitafiber system, with no cells.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8
12
16
20
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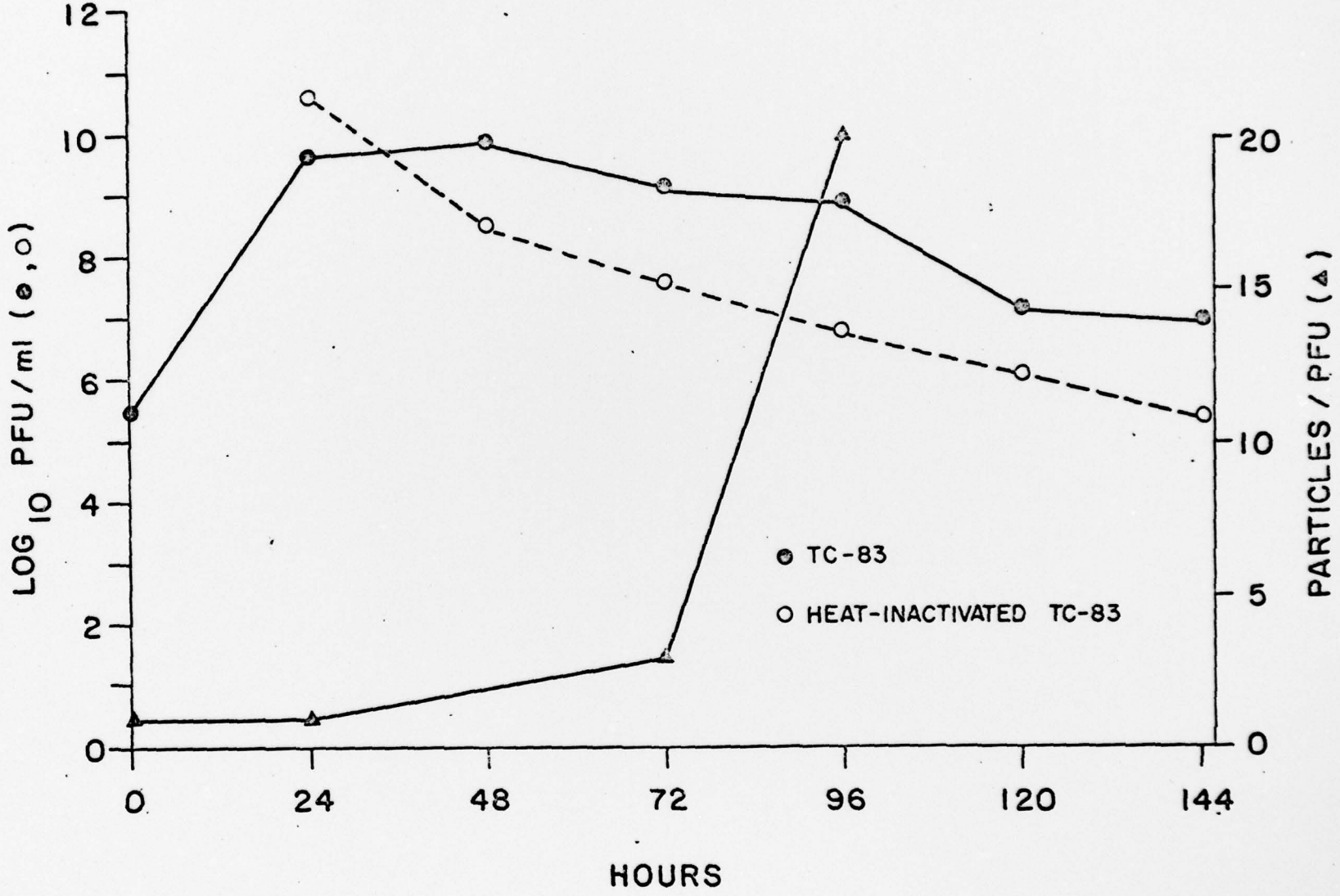
HOURS POSTINFECTION

1 μ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8
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15
10
5
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PARTICLES / PFU (▲)
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48
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96
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144
HOURS
● TC-83
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